In Vitro Cell. Dev. Biol.—Plant 40:608–612, November–December 2004
DOI: 10.1079/IVP2004584

1054-5476/04 $18.00+0.00

FUNGAL ENDOPHYTES INTRINSICALLY ASSOCIATED WITH MICROPROPAGATED PLANTS REGENERATED FROM NATIVE *BOUTELOUA ERIOPODA* TORR. AND *ATRIPLEX CANESCENS* (PURSH) NUTT.

JERRY R. BARROW*, PEDRO OSUNA-AVILA, AND ISAAC REYES-VERA

USDA, Agricultural Research Service, Jornada Experimental Range, P.O. Box 30003, MSC 3JER, NMSU, Las Cruces, NM 88003-8003

(Received 9 April 2004; accepted 2 August 2004; editor R. J. Newton)

Summary

Black grama (*Bouteloua eriopoda*) and fourwing saltbush (*Atriplex canescens*) are important grass and shrub species in arid rangelands of the northern Chihuahuan Desert. They are naturally colonized by dark septate endophytic fungi that cannot be eliminated by seed disinfestation. Plants were regenerated from both species and appeared to be fungus-free in axenic cultures. Analysis of callus and regenerated plants of both species using dual staining with light and scanning electron microscopy revealed fungal endophytes intrinsically associated with cells, roots and leaves of regenerated plants that are also associated with native plants. Fungal layers and biofilms prevent direct exposure of callus, root or leaf tissues to the external environment. Micropropagation is a valuable tool for identifying key fungal endophytes that enhance drought tolerance in native desert plants.

Key words: arid; biofilms; drought; mycorrhizae; symbiosis.

Introduction

Fungi have been detected in samples of every plant species examined to date (Berbee, 2001). They live within tissues of all plant species and aided in their initial invasion into terrestrial ecosystems (Blackwell, 2000). These relationships vary from pathogens and saprophytes to beneficial mutualists that enhance ecological fitness of plants in native ecosystems (Smith and Read, 1997). Surprising levels of fungal diversity were recently reported in leaves of native trees and in roots of a native grass (Arnold et al., 2000; Vandenkoornhuyse et al., 2002). Asymptomatic fungal endophytes inhabit the apoplastic spaces of above-ground plant parts of cool-season grasses and are vertically transmitted by seed (Clay and Schardl, 2002). Barrow et al. (1997) found that roots of native grasses and shrubs in the warm semiarid rangelands of the southwestern USA were primarily and extensively colonized by dark septate endophytic (DSE) fungi. Barrow and Aaltonen (2001) and Barrow (2003), using dual-staining methodology, analyzed DSE colonization of *Atriplex canescens* (Pursh) Nutt. (ATCA) and *Bouteloua eriopoda* Torr. (BOER), important forage shrub and grass species of the northern Chihuahuan Desert. They found that fungal incidence was much greater than previously thought, and that these fungi form a variety of previously unreported atypical and morphologically variable structures. DSE fungi systemically inhabit the entire apoplastic network, form inter- and intracellular structures with cells in both below- and above-ground structures, and are vertically transmitted by seed.

*Author to whom correspondence should be addressed: Email jbarrow@nmsu.edu

Meristem cultures and micropropagated plants have been used to produce plants free of fungi, bacteria, and viruses (Grum et al., 1998; Borkowska, 2002). Unable to produce endophyte-free BOER and ATCA plants by surface disinfestation of seed, we regenerated ATCA plants from meristem cultures and shoot regeneration and BOER plants via somatic embryogenesis. Cells and tissues were examined with scanning electron microscopy (SEM) and were dual stained and analyzed with high-magnification, differential interference microscopy to determine the presence of DSE colonization.

Materials and Methods

Micropropagation of BOER plants via somatic embryogenesis. Seeds of BOER were harvested from native populations on the USDA-ARS Jornada Experimental Range in the northern Chihuahuan Desert. They were surface-disinfested and germinated on MS (Murashige and Skoog, 1962) medium gelled with agar. Seedling shoots were excised and cultured on MS medium, supplemented with 4.52 μM Dicamba, under light, to induce callus and embryogenic structures. After transferring to MS auxin-free medium, embryo development progressed from globular, torpedo to mature embryos phenotypically identical to those naturally produced in seed (Osuna and Barrow, 2004).

Micropropagation of ATCA plants via shoot multiplication. ATCA seeds were surface-disinfested for 1 min in 95% ethanol, 7 min in 2.6% sodium hypochlorite (50% dilution of commercial liquid bleach), and rinsed three times in sterile distilled water. They were placed on hormone-free, modified White's media for germination (White, 1934). Intact seedlings were transferred to shoot regeneration medium consisting of MS basal salts with 11.42 μM indole-3-acetic acid (IAA) and 18.58 μM 6-furfurylaminopurine (kinetin). The medium was modified using the L2 vitamin formulation of Phillips and Collins (1979) with 30 $g\,l^{-1}$ sucrose and solidified with 0.8% agar. The pH was adjusted to 5.8 $\pm$ 0.05 for all media prior to autoclaving at 121°C at 125 kPa for 35 min. Cultures were grown in 100 $\times$ 25 mm

polystyrene Petri dishes and sealed with Parafilm®. Regenerated shoots were subcultured to fresh media every 4 wk.

Regenerated shoots were hyperhydric and were reverted to normal by transferring them to shoot proliferation medium modified by exchanging NH_4NO_3 with 4.40 g l^{-1} of casein hydrolyzate to compensate for the total nitrogen content of the standard MS formulation. Vitamin supplements were added according to the L2 formulation by Phillips and Collins (1979) plus 30 g l^{-1} sucrose and solidified with 5.0 g l^{-1} of Agargel® (Sigma Chemical Co., St Louis, MO), a blend of agar and phytagel documented to control hyperhydricity (Pasqualetto et al., 1986). Growth regulator composition consisted of 24.61 μ*M* 6-(γ-γ-dimethylallylamino)purine (2iP). The pH was adjusted and medium was autoclaved as noted above and dispensed in polycarbonate Magenta® GA7 vessels (Magenta Corp., Chicago, IL). Culture boxes were closed with vented Magenta® GA7 vessel covers with a 10 mm polypropylene membrane (0.22 μm pore size) (Magenta Corp.). All cultures were incubated at 28 ± 1°C under continuous fluorescent light (14–18 μmol m^{-2} s^{-1}).

Rooting of ATCA shoots. Normal shoots were transferred to White's medium (White, 1934) containing 2.46 μ*M* indolebutyric acid (IBA), 30 g l^{-1} sucrose and solidified with 2.5 mg l^{-1} of Phytagel® (Sigma Chemical Co.). The pH was adjusted and medium was autoclaved as above and dispensed in 107 × 107 × 96 mm-high, Lifeguard®, polycarbonate culture boxes closed with vented lids with an opening of 22 mm and a 0.3 μm pore size (Osmotek Ltd., Israel). Shoot explants, three nodes long, were excised by cutting at the lowest node. Rooted shoots were transferred to soil.

Tissue staining and analysis. Staining methods developed by Bevege (1968), Brundrett et al. (1983), Kormanik et al. (1980), and Phillips and Hayman (1970) were modified by Barrow and Aaltonen (2001) and Barrow (2003) for optimal fungal expression. Cells from suspension cultures, callus, roots and leaves from micropropagated plants were cleared in the autoclave in 2.5% KOH by increasing the temperature to 121°C over 5 min, maintained for 3 min and removed from the autoclave after 8 min. Samples were rinsed in tap water, placed in 1% HCl for 3 min at room temperature and rinsed for a further 3 min in dH_2O. They were dual stained in trypan blue (TB) (0.5 g TB in 500 ml glycerol, 450 ml dH_2O, and 50 ml HCl) and Sudan IV (SIV) (3.0 g SIV in 740 ml of 95% EtOH plus 240 ml dH_2O) and autoclaved at 121°C for 3 min. Stained tissues were rinsed for 3 min in dH_2O. Tissues were mounted on slides in permanent mounting medium (Peramount). A coverslip was placed over cells, calluses, root and leaf sections and pressed firmly to facilitate analysis at high magnification. Analysis was done with a Zeiss Axiophot microscope using both conventional and differential interference contrast DIC optics at 1000×. Callus and regenerated plants were also analyzed by scanning electron microscopy.

Results and Discussion

Initially, axenically cultured seedlings and regenerated plants of BOER and ATCA from surface-disinfested seed were presumed to be free of fungi because they were not visually evident nor did they grow on the culture medium. Their presence was microscopically verified in cell cultures and micropropagated plants, contradicting the assumption that such plants are microbe-free (Borkowska, 2002). Stained fungal tissue was consistently observed in cells of callus and suspension cultures (Fig. 1*a*, sf). Similar staining patterns were also observed with cells and tissues of germinating seedlings and native plants. After extensive studies, we considered these structures to be cohesively bound to the outer plasmalemma of the host cell (Barrow, unpublished). This intimate association insures its distribution to daughter cells and its subsequent vertical transmission by seed. Callus cultures of BOER (Fig. 1*b*, c) undergoing somatic embryogenesis (es) appeared to be free of fungi. However, SEM analysis revealed that both embryonic structures (Fig. 1*c*, es) and callus (c) were completely encapsulated within a fungal network and a mucilaginous biofilm (bf). Pirttila et al. (2002) also found that callus cultured from *Pinus sylvestris* L. buds was densely colonized and encapsulated within a similar biofilm. *In situ* hybridization further confirmed endophyte presence in 45% of their cultures. Suspension and callus cultures and several hundred micropropagated plants of BOER and ATCA have been found without exception to be colonized (data not shown).

Shoots (Fig. 1*d*, sh) developing from embryonic structures within the callus appeared to be devoid of fungi. However, stereomicroscopic examination of roots (rt) revealed branched hyphae (h) extending from the root surface. Examination of the developing plantlets (Fig. 1*e*) by SEM revealed that embryonic shoots (sh), roots (rt) and callus were totally encapsulated with a fungal biofilm (bf). SEM magnification of these root–fungus sections revealed a hyphal mass (Fig. 1*f*, h) covering the root surface encapsulated with a layer of biofilm (bf). Dual-stained, root–fungus sections analyzed by light microscopy revealed attached *Aspergillus conidiophores*. SEM analysis of embryonic leaf surfaces shown in Fig. 1*d* (sh) and Fig. 1*e* (sh) revealed parallel hyphae that formed a precisely organized pattern (Fig. 1*g*). Teliospores (tel) attached to the surface hyphae were also associated with stomata (st). A similar fungal pattern with regularly attached teliospores is consistently observed on native BOER leaves. Teliospores suggest that the leaf surface fungus belongs to the order *Uredinales* (Alexopoulos et al., 1996). Because native BOER grasses are known to be intermediate hosts for *Puccinia cacabata*, the leaf endophyte is likely a *Puccinia* sp. *Aspergillus ustus* has been isolated from native BOER plants and was found to be seed borne in ATCA (Barrow and Osuna, 2002). This indicates that two key fungal endophytes consistently associated with native BOER and ATCA plants are also intrinsically and inseparably associated with cells and micropropagated plants. The composite nature of plant and fungal cells has implications at the genetic, cell and physiological levels.

It was surprising that fungi were profusely associated with cells, roots, and leaves of cultured plants but failed to grow on the carbon-mineral-supplemented culture medium. This would be expected for *Puccinia* sp., obligate fungi that generally cannot be cultured independent of the host (Alexopoulos et al., 1996). On the other hand, *Aspergillus* sp. are ubiquitous saprophytic soil fungi that utilize a wide variety of exogenous carbon sources and are readily cultured on artificial media (Alexopoulos et al., 1996). In a few cases, after eventual death of micropropagated plants, *A. ustus* could be isolated from necrotic tissue and was cultured and maintained on the regeneration medium. For the most part, however, fungi from colonized root segments could not be induced to grow when plated on the culture medium. This suggests a selective allegiance to living host cells in its endophytic phase, and only after death or other drastic physiological impairment to the host cell was the fungus able to be isolated and cultured. Pathogens and mycorrhizal fungi may be eliminated from micropropagated plants, but in contrast these key endophytes are inconspicuously and intrinsically associated with living host tissue.

SEM analysis of micropropagated ATCA plant leaves revealed a uniquely different fungal pattern on the epidermis. These fungal structures are tightly packed, bladder cells (Fig. 2*a*, bc). Closer examination of bladder cells (Fig. 2*b*, bc) revealed branched internal hyphae (h). Native plant leaves of ATCA are also completely covered with bladder cells. Bladder cells of micropropagated plants cultured in a saturated environment are inflated while those on native plants under extreme drought are deflated and cover the entire leaf surface except for the stomata. The identity of the protective leaf fungus in ATCA is unknown.

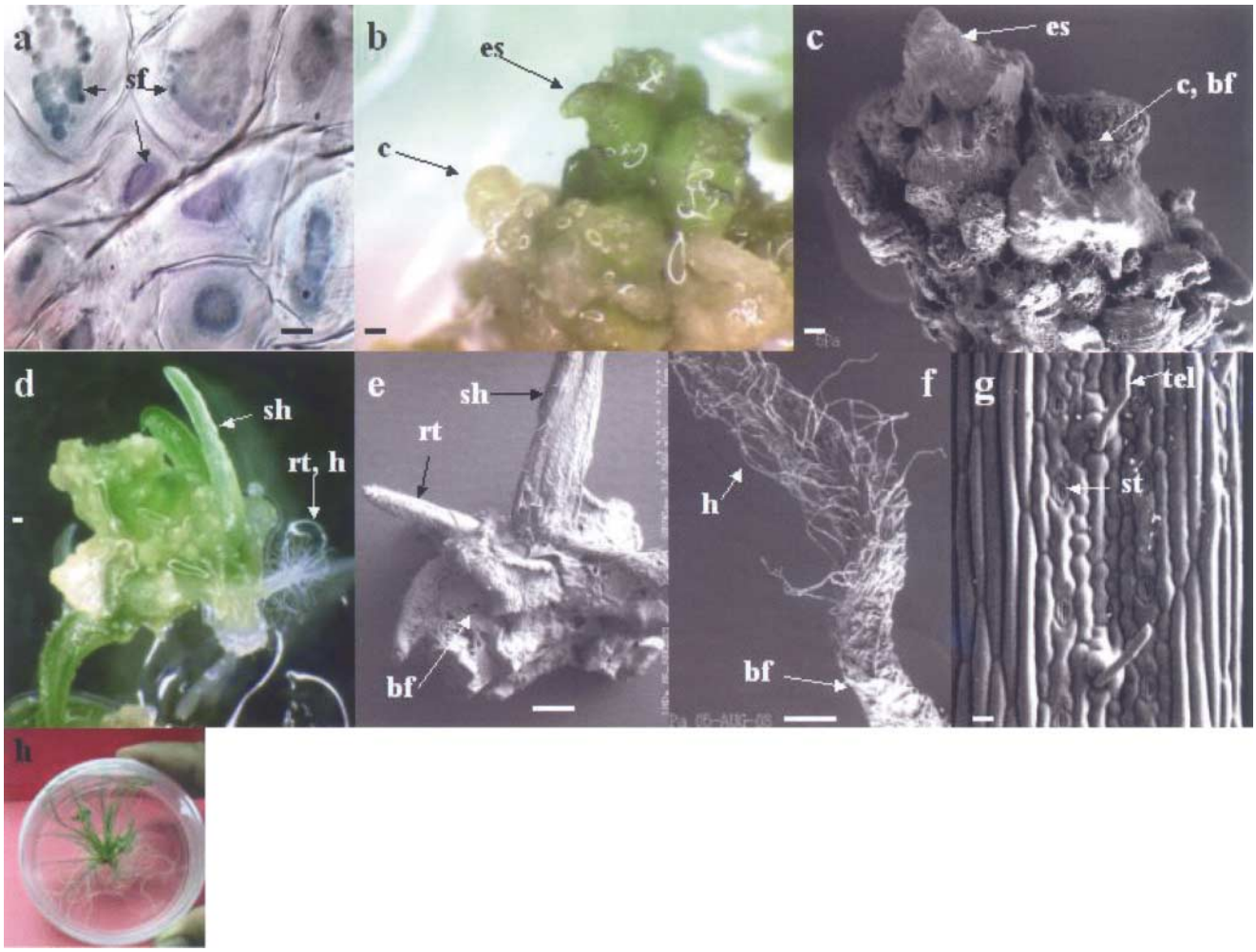

FIG. 1. *a*, Dual-stained fungal structures (sf) in *Bouteloua eriopoda* callus cultures. *b*, Embryogenic structures (es) regenerating from callus (c) of *B. eriopoda* (*bar* = 5 μm). *c*, An SEM micrograph of embryonic structures (es) developing from callus (c). The entire mass of cells and structures is covered with a fungal biofilm (bf) (*bar* = 20 μm). *d*, Embryonic shoots (sh) of *B. eriopoda* developing via somatic embryogenesis. Branched hyphae (h) extending from the root (rt) surface (*bar* = 200 μm). *e*, An SEM micrograph of an embryonic shoot (sh), root (rt), and callus all coated with a protective biofilm (bf) (*bar* = 200 μm). *f*, An SEM micrograph of a micropropagated root with a dense hyphal mass (h) covering the root surface. A biofilm (bf) encapsulates the hyphae and root (*bar* = 250 μm). *g*, An SEM micrograph of an embryonic shoot. Hyphae are arranged in tightly fitted, parallel rows and connected to stomata (st); fungal teliospores (tel) are attached (*bar* = 5 μm). *h*, A *Bouteloua eriopoda* plantlet, regenerated via somatic embryogenesis from embryonic shoot meristem cells of germinating seedlings.

We observed fungal associations with cells of sexually produced embryos in BOER seed (not shown). Meristematic cells of embryonic shoots were likewise colonized, resulting in colonized micropropagated plants. As plant cells differentiate into the various root and leaf tissues, associated fungi also assume morphological differentiation (not shown). Similar trends were observed in developing ATCA plantlets. This variation is illustrated in a dual-stained, micropropagated ATCA plant leaf. Stained fungal structures (Fig. 2*c*, fs) with attached lipid bodies occupy the photosynthetic cells (pc), while the lignified xylem (x) also stains positively (blue) for fungal tissue cohesively bound to the xylem rings. Such an integrated fungal structure with the xylem could be involved in active water transport by the fungus during extended drought periods. We conclude that the fungi function differently depending upon the type of host cell and its physiological state.

Stereomicroscopy of micropropagated ATCA roots revealed dense external hyphae (Fig. 2*d*, h) similar to the external hyphae of mycorrhizal fungi. SEM analysis of the root fungus structures revealed a dense hyphal mass completely covering the surface, similar to that of micropropagated BOER roots (Fig. 2*e*). Figure 2*f* shows embryonic ATCA leaf and root tissues developing from callus. Bladder cells (Fig. 2*f*, bc) are developing on leaf tissue, while a dense hyphal mass with biofilm (bf) totally encapsulates the callus and root surface (rt).

Comparing cells and tissues of micropropagated BOER and ATCA plants with those from native populations revealed key fungal endophytes that are intrinsically associated with all cells. These results are consistent with those of Pirttila et al. (2003) who isolated two endophytic fungal species from pine tissue cultures. Using probes that targeted the 18S rRNA of *Hormonema dematiodes*

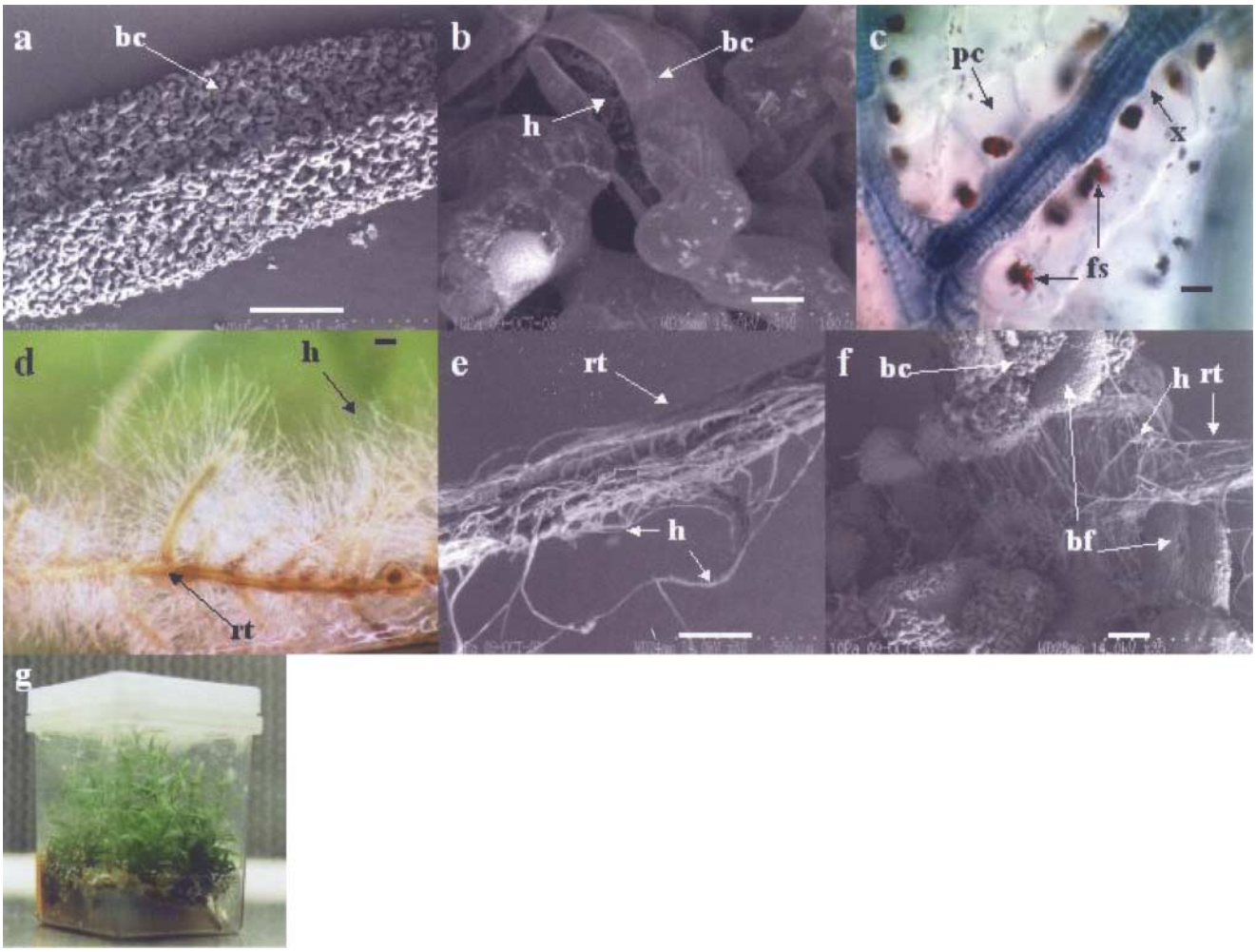

FIG. 2. *a*, An SEM micrograph of a micropropagated *Atriplex canescens* leaf covered with tightly fitted, fungal bladder cells (bc) (*bar* = 500 μm). *b*, An SEM micrograph of a bladder cell (bc) showing internal branched hyphae (h) (*bar* = 5 μm). *c*, A dual-stained leaf from a micropropagated plant. Trypan blue-stained fungal structures (fs) occupy the photosynthetic bundle sheath cells (pc). Xylem stains positively for fungal tissue (*bar* = 10 μm). *d*, A stereomicrograph of a micropropagated *A. canescens* root. Dense external hyphae extend from the surface (*bar* = 250 μm). *e*, An SEM micrograph of a micropropagated *A. canescens* root showing a dense hyphal (h) matrix on the surface (*bar* = 250 μm). *f*, An SEM micrograph of *A. canescens* plantlets regenerating from callus. Bladder cells (bc) are forming on embryonic leaves; hyphae (h) are observed covering callus and developing root (rt) (*bar* = 250 μm). *g*, *Atriplex canescens* regenerated from shoot meristems.

and *Rhodotorula minuta*, they found that *R. minuta* was associated with meristematic cells in 40% of the samples, while *H. dematiodes* was not found in meristem cells but was localized in scale tissues of cultured buds. Also significant is the observation that none of these plant cells or tissues are directly exposed to the external environment, whether it is the highly controlled or the harsh external environment.

While some pathogenic and symbiotic fungi may be removed from host plants by micropropagation, this study shows that some key endophytes intrinsically associated with host plants may not be separated by axenic culture. It is generally accepted that endophytic and mycorrhizal fungi enhance drought tolerance in their respective hosts. However, the mechanisms are not currently understood. Fungi may be ideal symbiotic partners because they and other microorganisms characteristically produce mucopolymeric substances, biofilms, that have an important protective role in extreme environments (Costerton et al., 1999; Krembs et al., 2002). Significantly, these key endophytes prevent direct exposure of callus, root, and shoot tissues to the external environment. Microbial biofilm matrices are complex and are analogous to organized tissues of higher organisms. Composed of polysaccharides, they represent a substantial organic carbon component of tissue surfaces and the rhizosphere and buffer encapsulated organisms from dessication. Their high propensity for water absorption suggests that they provide a saturated microenvironment that would protect both plants and associated microbes from desiccation in arid environments where both roots and shoots are chronically exposed to extreme soil and aerial environments. We believe that micropropagation is a valuable tool in identifying key fungal endophytes and studying these unique relationships and how they interact at the genetic, cell, and physiological levels.

Micropropagation of both BOER and ATCA yielded ample quantities of regenerated plants for microscopic studies. More than 100 intact plantlets were obtained from callus induced of each

seedling (Fig. 1*h*). Somatic embryos were only induced for 6 mo. after callus induction. Consistent with the findings of Mei et al. (1997), we found that meristem cultures profusely produced multiple shoots (Fig. 2*g*), of which 56–88% could be rooted and transplanted into soil. Most regenerated plants were sacrificed for microscopic studies. Fungal colonization was consistent in all regenerated BOER and ATCA plants.

References

Alexopoulos, C. J.; Mims, C.; Blackwell, W. M. Introductory mycology, 4th edn. New York: Wiley; 1996:575–587.

Arnold, A.; Maynard, Z.; Gilbert, G. S.; Coley, P. D.; Kursar, T. A. Are tropical fungal endophytes hyperdiverse? Ecol. Lett. 3:267–274; 2000.

Barrow, J. R. Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in southwestern USA rangelands. Mycorrhiza 13:239–247; 2003.

Barrow, J. R.; Aaltonen, R. E. A method of evaluating internal colonization of *Atriplex canescens* (Pursh) Nutt. roots by dark septate fungi and how they are influenced by host physiological activity. Mycorrhiza 11:199–205; 2001.

Barrow, J. R.; Havstad, K. M.; McCaslin, B. D. Fungal root endophytes in fourwing saltbush, *Atriplex canescens*, on arid rangelands of southwestern USA. Arid Soil Res. Rehab. 11:177–185; 1997.

Barrow, J. R.; Osuna, P. Phosphorus solubilization and uptake by dark septate fungi in fourwing saltbush, *Atriplex canescens* (Pursh) Nutt. J. Arid Environ. 51:449–459; 2002.

Berbee, M. L. The phylogeny of plant and animal pathogens in the Ascomycota. Physiol. Mol. Plant Pathol. 59:165–187; 2001.

Bevege, D. I. A rapid technique for clearing tannins and staining intact roots for detection of mycorrhizas caused by *Endogone* spp., and some records of infection in Australian plants. Trans. Br. Mycol. Soc. 51:808–810; 1968.

Blackwell, M. Enhanced: terrestrial life – fungal from the start? Science 289:1884–1885; 2000.

Borkowska, B. Growth and photosynthetic activity of micropropagated strawberry plants inoculated with endomycorrhizal fungi (AMF) and growing under drought stress. Acta Physiol. Plant. 24:365–370; 2002.

Brundrett, M. C.; Piche, Y.; Peterson, R. L. A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can. J. Bot. 62:2128–2134; 1983.

Clay, K.; Schardl, C. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am. Nat. 160:S99–S127; 2002.

Costerton, J. W.; Stewart, P. S.; Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322; 1999.

Grum, M.; Camloh, M.; Rujdolph, K.; Ravnikar, M. Elimination of bean seed-borne bacteria by thermotherapy and meristem culture. Plant Cell. Tiss. Organ Cult. 52:79–82; 1998.

Kormanik, P. P.; Bryan, W. C.; Schultz, R. C. Procedures and equipment for staining large numbers of plant root samples for endomycorrhizal assay. Can. J. Microbiol. 26:536–538; 1980.

Krembs, C.; Eicken, H.; Junge, K.; Deming, J. W. High concentrations of exopolymeric substances in Arctic winter sea ice: implications for the polar ocean carbon cycle and cryoprotection of diatoms. Deep-Sea Res. Part I 49:2163–2181; 2002.

Mei, B.; No, E. G.; McWillliams, E. L.; Gould, J. H.; Newton, R. J. *In vitro* regeneration of fourwing saltbush [*Atriplex canescens* (Pursh) Nutt.]. J. Range Manage. 50:413–418; 1997.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Osuna, P.; Barrow, J. R. Regeneration of black grama (*Bouteloua eriopoda* Torr. Torr). In Vitro Cell. Dev. Biol. Plant 40:299–302; 2004.

Pasqualetto, P. L.; Zimmerman, R. H.; Fordham, I. Gelling agent and growth regulator effects on shoots vitrification of 'Gala' apple *in vitro*. J. Am. Soc. Hort. Sci. 111:976–980; 1986.

Phillips, G. C.; Collins, G. B. *In vitro* tissue culture of selected legumes and plant regeneration of red clover. Crop Sci. 19:59–64; 1979.

Phillips, J. M.; Hayman, D. S. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55:158–161; 1970.

Pirttila, A. M.; Laukkanen, H.; Hohtola, A. Chitinase production in pine callus (*Pinus sylvestris* L.): a defense reaction against endophytes. Planta 214:848–852; 2002.

Pirttila, A. M.; Pospiech, H.; Laukkanen, H.; Myllyla, R.; Hohtola, A. Two endophytic fungi in different tissues of Scots Pine buds (*Pinus sylvestris* L.). Microb. Ecol. 45:53–62; 2003.

Smith, S. E.; Read, D. J., eds. Mycorrhizal symbiosis, 2nd edn. San Diego, London: Academic Press; 1997:59–60.

Vandenkoornhuyse, P.; Baldauf, S. L.; Leyval, C.; Straczek, J.; Young, P. W. Extensive fungal diversity in plant roots. Science 295:2051; 2002.

White, P. R. Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. 9:585–600; 1934.